IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Lusso et al. Art Unit: 1648
Serial No.: 10/524,549 Examiner: Li, Bao Q
Filed: September 13, 2005
Title: *Pharmaceutical Composition Comprising an HIV Envelope Protein and CD4*

Commissioner for Patents
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DECLARATION OF SAMUELE BURASTERO UNDER 37 C.F.R. §1.132

I, **Samuele Burastero** declare:

1. I am the co-inventor of the subject matter described and claimed in the above-captioned patent application. I directly participated, together with Paolo Lusso, to the conception and to the implementation of the project which brought us to develop the processes and the products described in the above captioned application

2. I have reviewed the Office Action mailed October 3, 2007 in the above-captioned application.

3. The following experiments were conducted to further characterize the anti-CD4 antibodies raised from mice immunized with cell-membrane expressed, Env-CD4 molecular complexes. We have been studying entry inhibitors of HIV in the model of exposed uninfected individuals. In a subset of subjects who display natural resistance to infection, anti-CD4 antibodies were correlated to disease protection in cohorts of individuals with different routes of exposure and living in different countries. These antibodies display broad spectrum of neutralization and the molecular components they recognize were not affected by mutations generated by the viral escape mechanisms. As such, mouse model strategies were set up and aimed to selectively enrich immunogens with epitopes generated by viral and cellular molecules following the docking of HIV to target cells, which were expected to generate antibodies with binding activity resembling those identified in HIV-exposed uninfected individuals.

4. Mice were immunized using cell surface expressed native HIV-1 gp160 complexed with a truncated form of human CD4. Balb/c mice were injected with soluble CD4 (sCD4)-bound HIV-1 Env (gp41+gp120) expressed in the "native", oligomeric form

on autologous murine fibroblasts (NIH 3T3) by recombinant vaccinia technology. The selection strategy used to screen sera and hybridomas included the ability to bind preferentially to the immobilized sCD4/gp120 complex in ELISA. Membrane expression of transiently exposed CD4-gp120 complex-dependent epitopes was checked by reactivity with CD4i mAbs (17b, 48d, D19). The fusion competence of the complexes was demonstrated using a vaccinia-based fusion assay with target cells expressing the relevant coreceptor (in the absence of membrane CD4). Hybridomas were generated and selected from mice with high titers of anti-complex antibodies. Monoclonal antibodies were derived by standard protocols from several somatic cell fusions and screened on the basis of binding to solid-phase human CD4 or gp120 and preferred binding to CD4-gp120 complexes. One clone (DB-81) was characterized and expanded for further studies. See Figure 1. Sequences of the variable parts of the IgG1, k murine monoclonal DB-81 have been recently deposited and are publicly available through EMBL (AM944499 and AM944500).

5. Flow cytometry analysis confirmed that mAb DB-81 (IgG1, k) preferentially recognized soluble recombinant CD4 bound to the native, oligomeric form of gp120 expressed on the surface membrane of PM-1 cells chronically infected with the primary R5 isolate Ba-L (Figure 2, top panel). In reciprocal experiments, an increased binding of mAb DB-81 was observed after treating uninfected PM-1 cells (that express high levels of CD4) with soluble gp120 derived from different HIV-1 isolates (i.e., IIIB [X4] or Ba-L [R5]), as compared to binding to untreated PM-1 (Figure 2, bottom panel). ELISA assays showed that binding of mAb DB-81 to solid-phase human 2-domain (2D)-sCD4 or 4D-sCD4 was increased by pre-incubation with recombinant gp120 derived from different viral strains (e.g., Ba-L, IIIB)(data not shown).

6. To better quantify the gain in binding activity achieved upon CD4/gp120 complex formation, the affinity of DB-81 for the CD4-Env complex was characterized with a plasmon resonance approach. When using different gp120, the DB-81 affinity for the complex was consistently found to be three orders of magnitude above the affinity for CD4 alone (10^{-11} versus 10^{-8} M, respectively). In the case of Env gp120 Bal, DB-81 bound the CD4 with an affinity of 8.57 nM x 10^{-9} M, whereas when the CD4 was

complexed with the gp120, the affinity increased to 9.60 pM) (i.e., 892 times) (Figure 3). From the kinetics of binding, it appears that that the parameter responsible for this dramatic change was not the association constant, that is quite high for both reactants (around 10^5 $M^{-1}s^{-1}$), but the dissociation constant, which is in the 10^{-3} s^{-1} and 10^{-6} s^{-1} range for CD4 and for the complex, respectively. These results highlight the striking difference between DB-81 and an anti-CD4 mAb, which has been recently proposed for anti-HIV passive immunotherapy (TNX-355® from Tanox). This data also indirectly indicates that the DB-81 mAb displays its anti-viral activity through a complementary, non-overlapping mechanism, which selectively targets post-binding, conformation-dependent molecular sites. Previously performed functional affinity studies, which used classical immunochemistry approaches, provided similar results. Cross-inhibition studies performed by both cytofluorimetry and ELISA showed no reciprocal binding competition between mAb DB-81 and other CD4-specific monoclonals, including mAb 55, OKT4, OKT4A, Leu3a, SIM-1 and SIM-4 (not shown).

7. To study the ability of mAb DB-81 to block infection with HIV-1, different test systems were used. First, an HIV-1 envelope-mediated cell fusion assay that employs, as effector cells, chronically infected PM-1 cells expressing different HIV-1 Envs in their native, fusion-competent conformation was used. Both laboratory-adapted and primary isolates of different coreceptor-usage phenotype were tested, including Ba-L (R5), B117 (R5X4), IIIB and 6195 (both X4) (Figure 4, panel A). In these assays, mAb DB-81 efficiently inhibited HIV-1 envelope-mediated cell fusion in a dose-dependent fashion. The potency of fusion inhibition was similar to that observed with equimolar concentrations of mAb Leu3A (an anti-CD4 mAb that specifically recognizes the gp120-binding site and potently blocks CD4/gp120 binding) (Figure 4, panel B). Pre-adsorption experiments showed that the fusion-inhibition activity was more efficiently absorbed by pre-adsorption of mAb DB-81 on solid-phase CD4 complexed with Ba-L or IIIB gp120 than by CD4 alone (Figure 4, panel C). DB81 also efficiently inhibited peripheral blood mononuclear cells based HIV-1 neutralization assay with both lab-adapted (e.g., IIIB and Ba-L) and primary isolates.

Neutralization of different HIV-1 isolates by MAb DB81was evaluated in

phytohemagglutinin-activated human PBMC, previously treated or not with mAb DB81 at different concentrations. PBMC were acutely infected with isolates IIIB (X4), 92US077 (R5X4), BaL (R5), or 91US714 (R5). The DB81 mAb was maintained at the same concentrations throughout the entire experiment. The levels of p24 antigen in the culture supernatants were determined by ELISA at day 5 post-infection. Results are reported as the extrapolated mAb concentration which was capable of 50% inhibiting the baseline p24 production (ID50 p24) in the following Table 1.

Table 1.

strain	coreceptor	ID50 p24 µg/ml
IIIB	X4	0.3
Ba-L	R5	0.4
92US077	X4	1.2
92US714	R5	0.9

Taken together, these results indicate that the DB-81 epitope is accessible in assays where oligomeric gp120 is involved and is present in CD4/Env complexes derived from multiple HIV strains.

8. To verify the potential immunosuppressive effects of mAb DB-81, *in vitro* assays were used. In primary human PBMC proliferation assays to tetanus toxoid or to polyclonal activators, mAb DB-81 displayed no significant interference with T-cell proliferation when continuously kept in culture, whereas equimolar concentrations of mAb Leu3a was potently suppressive (Figure 5). In experiments of CD4 down-modulation using both PM-1 cells and primary PBMC, mAb DB-81 caused a very limited down-modulation of cell surface CD4, unlike mAb Leu3a that induced a rapid a dramatic loss of cell surface signal as assessed using the non competing mAb OKT4 (not shown). Although complement fixation is expected to be influenced by the isotype of the humanized derivative of this monoclonal, DB-81 was *per se* poorly efficient in complement fixation, as measured by propidium iodide incorporation following

incubation with rabbit complement, as compared to the isotype-matched mAb Leu3a (Figure 6).

9. Cross-inhibition experiments were performed by measuring the binding of 1 µg/ml biotinylated mAb DB-81 to solid-phase CD4 in the absence or in the presence of 10 µg/ml of competitor monoclonal antibodies. Binding of DB-81 was subsequently revealed with strepto-avidin-alkaline phosphatase conjugated (+ substrate). Inhibition of binding below 20% of control was observed with mAb 55, OKT4, OKT4A, Leu3a, SIM-1 and SIM-4. Similarly, no inhibition (i.e., no inhibition above 15% of control) was observed in cytofluorimetric assays where the same relative concentrations (1 to 10) of ligand and competitor were used, and streptoavidin-FITC conjugated was used to reveal DB-81 binding to PM-1 CD4 positive cells. Results are summarized in the following table (Table 2).

Table 2

	Percent inhibition	
Inhibitor	**ELISA**	**Flow cytometry**
Leu 3a	-2.0	3.8
OKT4	1.0	-4.0
mAb 55	11.0	9.4
SIM2	6.0	5.0
SIM4	4.4	7.1
DB-81	94.0	78.0
OKT3	4.0	1.0

These results do not provide a precise mapping of the binding site of the monoclonal. OKT4 and Leu3A bind to the first domain of CD4. However, direct binding experiments with 2-domains recombinant soluble CD4 (from AIDS Reagent Program) and with a CD4-IgG complex (previously known as "immunoadhesin") (Genentech) show that the DB-81 binding site is located within the first two domains of the CD4 molecule.

10. All statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: April 10, 2008

Samuele Burastero

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